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I HEREBY CERTIFY that annexed hereto is a true copy of documents filed in connection with the following patent application:

Application No.	1999/0033
Date of Filing	15 January 1999
Applicant	ENTERPRISE IRELAND (trading as BioResearch Ireland) a body established under the Irish Industrial Development (Enterprise Ireland) Act, 1998 of Glasnevin, Dublin 9, Ireland, and UNIVERSITY COLLEGE CORK, a body organised and existing under Charter and being a constituent college of the National University of Ireland, of College Road, Cork, Ireland.

Dated this 9 day of December 2003.

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FORM NO. 1

990033

REQUEST FOR THE GRANT OF A PATENT

PATENTS ACT, 1992

The Applicant(s) named herein hereby request(s)

[x] the grant of a patent under Part II of the Act

[ ] the grant of a short-term patent under Part III of the Act

on the basis of the information furnished hereunder.

1. Applicant(s)

Name: ENTERPRISE IRELAND (trading as BioResearch Ireland)

Address: Glasnevin,
Dublin 9,
Ireland.

Description/Nationality: A body established under the Irish Industrial Development (Enterprise Ireland) Act, 1998.

Name: UNIVERSITY COLLEGE CORK

Address: College Road,
Cork,
Ireland.

Description/Nationality: A body organised and existing under Charter and being a constituent college of the National University of Ireland.

2. Title of Invention

Bifidobacterium longum infantis in the treatment of inflammatory bowel disease.

3. Declaration of Priority on basis of previously filed application(s) for same invention (Sections 25 & 26)

Previous filing date	Country in or for which filed	Filing No.

CONTINUED OVER

4. Identification of Inventor(s)

Name(s) of person(s) believed
by Applicant(s) to be the inventor(s)

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5. Statement of right to be granted a patent (Section 17(2)(b))

The Applicants are assignees of the inventors by virtue of a Deed of Assignment dated January 14, 1999.

6. Items accompanying this Request - tick as appropriate

(i) [x] Prescribed filing fee (£100.00)

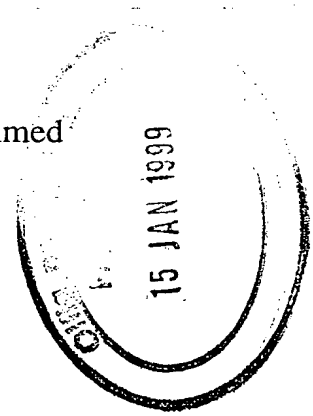
(ii) [x] Specification containing a description and claims
[ ] Specification containing a description only
[x] Drawings referred to in description or claims

(iii) [x] An abstract

(iv) [ ] Copy of previous application(s) whose priority is claimed

(v) [ ] Translation of previous application whose priority is claimed

(vi) [ ] Authorisation of Agent (this may be given at 8 below if this Request is signed by the Applicant(s))



7. Divisional Application(s)

The following information is applicable to the present application which is made under Section 24 -

Earlier Application No: .....
Filing Date: .....

990033

8. Agent

The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

Name	Address
ANNE RYAN & CO.	60 Northumberland Road, Ballsbridge, Dublin 4, Ireland.

9. Address for Service (if different from that at 8)

ANNE RYAN & CO., Authorised Patent Agents.

Signed Name(s): by: Anne Ryan

Capacity (if applicant is a body corporate):

Date January 15, 1999.

Bifidobacterium longum infantis in the treatment of inflammatory bowel disease

This invention relates to probiotic *Bifidobacterium* strains which have various applications in foodstuffs and in medicine. More particularly, the invention relates to probiotic strains of bifidobacteria which are capable of beneficially modifying and consequently alleviating observable symptoms in inflammatory bowel disease.

Consumers are becoming increasingly aware of matters which may be necessary for maintenance of their environment, health and nutrition. In response, scientific research has focussed upon the roles that diet, stress, and modern medical practices (e.g., antibiotics and radiotherapy) may play in threatening human health. In particular, population dynamics shifting towards older societies are increasing the incidence of illnesses which may be caused by deficient or compromised microflora such as gastrointestinal tract (GIT) infections, constipation, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) – Crohn's disease and ulcerative colitis, food allergies, antibiotic-induced diarrhoea, cardiovascular disease, and certain cancers (e.g., colorectal cancer).

Probiotics have been defined as live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance, or more broadly, as living micro-organisms, which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition. Cocktails of various micro-organisms,

particularly species of *Lactobacillus* and *Streptococccus*, have traditionally been used in fermented dairy products to promote health.

In recent years, the commercial manufacture and marketing of functional foods (foods which affect functions of the body in a targeted manner so as to bring about positive effects on physiology and nutrition), particularly probiotic (Acidophilus-Bifidus) yoghurts, has spread from the well-established Japanese niche market place into the lucrative and expanding European Union. While a number of probiotic bacteria of human origin are now being exploited commercially (e.g., *L. acidophilus* LA-1), many consumers, consumer organisations, and members of the scientific community are sceptical of such products and their publicised probiotic claims. The dairy-food industry is therefore under considerable pressure to scientifically validate these new probiotic food products.

Criteria which have been suggested for the selection of potentially effective probiotic micro-organisms may be summarised as follows: human origin, non-pathogenic behaviour, resistance to technological processes (i.e., viability and activity in delivery vehicles), resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to colonise the GIT, production of antimicrobial substances, ability to modulate immune responses, and the ability to influence metabolic activities (e.g., cholesterol assimilation, lactase activity, vitamin production) (Huis in't Veld J, Shortt C. Selection criteria for probiotic micro-organisms. In: Leeds, A.R., Rowland, I.R. eds. Gut Fora and Health – Past, Present and Future. London: The Royal Society of Medicine Press Ltd., 1996:19-26).

Bifidobacteria are one of several predominant culturable bacteria present in the colonic microflora.

The functions of endogenous bifidobacteria in the colon have not been completely elucidated. However, it is recognised that exclusively breast-fed infants have a reduced risk of diarrhoea compared with formula-fed infants. The fact that these infants have greater numbers of colonic bifidobacteria may in part explain this observed health advantage as the occupation of available niches in the GIT by large numbers of nonpathogenic bifidobacteria may help prevent bacterial infection. The pathogenesis of Crohn's disease is thought to be related to colonic bacterial microflora (Targan, S. and Shanahan, F. Inflammatory bowel disease: From bench to bedside. Williams and Wilkins 1994.) It has recently been found that patients suffering from active Crohn's disease have significantly less recoverable bifidobacteria in their faeces compared with healthy individuals. This reduction in bifidobacteria numbers was observed to be directly correlated with decreased levels of β-D galactosidase production and activity (Favier, C. *et al;* Dig. Dis. Sci. 1997;42:817-822). β-D galactosidase is an enzyme produced by bifidobacteria. These results support suggestions proposed in other studies that strains of bifidobacteria may play important roles in maintaining a balanced healthy intestinal microflora.

Bifidobacteria are considered to be probiotics as they are living organisms which exert health effects beyond basic nutrition when ingested in sufficient numbers. Numerous ingested bifidobacteria must reach the site of action in the gut in order to exert a probiotic effect. A

minimum level of approximately 10^6-10^7 viable bifidobacteria *per* gram intestinal contents has been suggested (Bouhnik, Y., Lait 1993;73:241-247). There are reports in the literature which show that *in vivo* studies completed in adults and in infants indicate that some strains of bifidobacteria are capable of surviving passage through the gastrointestinal tract. Significant differences have been observed between the abilities of different bifidobacteria strains to tolerate acid and bile salts, indicating that survival is an important criterion for the selection of potential probiotic strains.

Ingestion of bifidobacteria can improve gastrointestinal transit.

Furthermore, indirect evidence in humans demonstrates that consuming milk fermented by bifidobacteria can lead to reduced levels of certain faecal enzymes such as β–glucuronidase implicated in the conversion of procarcinogens to carcinogens (Bouhnik Y. *et al*; Eur. J. Clin. Nutr. 1996;50:269-273) Faecal-borne putrefaction metabolities such as *p*-cresol, indole and ammonia were also reduced when subjects consumed milk fermented by *Bifidobacterium longum* and *S. thermophilus* (Takiguchi, R. *et al. Bifidus – Flores, Fructus et Semina* 1996;9:135-140).

Antimicrobial activity has been reported to be associated with bifidobacteria. Also, bifidobacteria have been shown to modulate various parameters of the immune system.

Mucosal inflammation in IL-10 deficient mice has been reported to be reduced by feeding the subject animals a preparation of lactic acid bacteria (Madsen, K. *et al.* Gastroenterol. 1997;112:A1030.). Further studies completed in rats have demonstrated that ingestion of bifidobacteria can suppress aberrant crypt foci (early preneoplastic lesions) formation in the colon (Kulkarni, N. and Reddy, B. Proc. Soc. Experim. Biol. Med. 1994;207:278-283.) in addition to significant decreases in colon tumor incidence and in the numbers of tumors present (Singh, J. *et al* Carcinogenesis 1997;18:833-841).

There is an on-going search for probiotic strains with particular beneficial effects on nutrition and therapy and on health generally.

The invention provides a strain of *Bifidobacterium longum infantis* isolated from resected and washed human gastrointestinal tract which is capable of combating the effects of inflammatory bowel disease, said capability being maintained in the presence of physiological concentrations of human bile and human gastric juice.

The strain of *Bifidobacterium longum infantis* can be defined in terms of its capability to combat the effects of inflammatory bowel disease as measured by measuring a reversal of a wasting disease induced in severe combined immunodeficient recipient mice (SCID) which have been administered purified $CD4^+$, $CD45RB^{high}$ T cells.

The capability of the strain of *Bifidobacterium longum infantis* to combat the effects of inflammatory bowel disease can also be

measured by measuring the reduction in colonic inflammation in IL-10 deficient mice (IL-10^{+} 129 Svex strain) following administration of one or more of the strains of *Bifidobacterium longum infantis* according to the invention alone or in combination with a strain of *Lactobacillus salivarius* as hereinafter defined.

Interleukin 10 (IL-10) is an important regulatory cytokine that supresses effector functions of macrophage/monocytes, T helper 1 (Th1) cells, and natural killer cells. In addition, IL-10 augments proliferation and differentiation of B cells. Murine models lacking the IL-10 gene spontaneously develop inflammatory bowel disease and gastrointestinal tumors. The gastrointestinal flora have been implicated in the pathogenesis of these disease states as germ free animals do not develop disease.

Preferably, the strain of *Bifidobacterium longum infantis* has inhibitory activity against a broad range of Gram positive and Gram negative bacteria.

Further, preferably, the strain of *Bifidobacterium longum infantis* exhibits a broad-spectrum of activity against bacteria including *Staphylococcus*, *Pseudomonas*, *Coliform* and *Bacillus* species.

A particularly preferred strain of *Bifidobacterium longum infantis* is *Bifidobacterium longum infantis* strain UCC 35624 or a mutant or variant thereof.

A deposit of *Bifidobacterium longum infantis* strain UCC 35624 was made at The National Collections of Industrial and Marine Bacteria Limited (NCIMB) on January 13, 1999 and accorded the accession number NCIMB 41003.

The invention also provides a formulation comprising a strain of *Bifidobacterium longum infantis* as hereinbefore defined.

The formulation according to the invention can comprise two or more of said strains of *Bifidobacterium longum infantis*.

The formulation according to the invention can also comprise a strain of *Lactobacillus salivarius* isolated from resected and washed human gastrointestinal tract which inhibits a broad range of Gram positive and Gram negative micro-organisms and which secretes a product having antimicrobial activity into a cell – free supernatant, said activity being produced only by growing cells and being destroyed by proteinase K and pronase E, the inhibitory properties of said strain and its secretory products being maintained in the presence of physiological concentration of human bile and human gastric juice.

Such strains of *Lactobacillus salivarius* are disclosed in WO 98/35014.

An especially preferred strain of *Lactobacillus salivarius* is *Lactobacillus salivarius* strain UCC 118 or a mutant or variant thereof.

A deposit of *Lactobacillus salivarius* strain UCC 118 was made at the NCIMB on November 27, 1996 and accorded the accession number NCIMB 40829.

The strain of *Bifidobacterium longum infantis* according to the invention can be used in foodstuffs.

The strain of *Bifidobacterium longum infantis* according to the invention can also be used as a medicament.

The strain of *Bifidobacterium longum infantis* or a formulation according to the invention is particularly useful for combating the effects of inflammatory bowel disease, including Crohn's disease and ulcerative colitis.

In the accompanying drawings:

Fig. 1 is a graph of cfu/ml *versus* time for *Bifidobacterium longum infantis* strain 35612 as described in Example 2;

Fig. 2 is a graph of cfu/ml *verus* time for *Bifidobacterium longum infantis* strain 35624 as described in Example 2;

Fig. 3 is a graph of percentage weight change *versus* time (days) for five SCID mice (1-5) administered strain UCC 35624 as described in Example 5;

Fig. 4 is a graph of average percentage weight change *versus* time (days) for the SCID mice (1-5) administered strain UCC 35624 as described in Example 5;

Fig. 5 is a graph of percentage weight change *versus* time (days) for mice (6-10) administered a combination of strains UCC 118 and UCC 35624 as described in Example 5;

Fig. 6 is a graph of average percentage weight change *versus* time (days) for mice (6-10) administered a combination of strains UCC 118 and UCC 35624 as described in Example 5;

Fig. 7 is a graph of percentage weight change *versus* time (days) for mice (11-15) administered a combination of strains UCC 118 and UCC 35624 as described in Example 5;
and

Fig. 8 is a graph of average percentage weight change *versus* time (days) for mice (11-15) administered a combination of strains UCC 118 and UCC 35624 as described in Example 5;

The invention will be further illustrated by the following Examples.

Example 1

Isolation of probiotic bacteria

Appendices and sections of the large and small intestine of the human G.I.T., obtained during reconstructive surgery, were screened for probiotic bacterial strains as shown in Table 1.

Table 1

Gastrointestinal tract tissue samples screened for the presence of probiotic bacteria

Sample	Location
A	Ileum
B	Colon
C	Ileal-caecal region
D	Appendix
E	Appendix
F	Ileum
G	Ileal-caecal region

All samples were stored immediately after surgery at -80°C in sterile containers.

Frozen tissues were thawed, weighed and placed in cysteinated (0.05%) one quarter strength Ringers' solution. Each sample was gently shaken to remove loosely adhering microorganisms (termed - wash 'W'). Following transfer to a second volume of Ringers' solution, the sample was vortexed for 7 min to remove tightly adhering bacteria (termed -sample 'S'). In order to isolate tissue embedded bacteria, samples A, B and C were also homogenised in a Braun blender (termed - homogenate 'H'). The solutions were serially diluted (dilution 10^{-1} from a wash sample was labelled W1, dilution 10^{-2} was labelled W2 and the same labelling system was used for the 'S' and 'H' samples) and spread-plated (100µl) on to the following agar media: RCM (reinforced clostridial media) and RCM adjusted to pH 5.5 using acetic acid; TPY (trypticase, peptone and yeast extract), Chevalier, P. *et al.* (1990) *J. Appl. Bacteriol 68*, 619-624). MRS (deMann, Rogosa and Sharpe); ROG (acetate medium (SL) of Rogosa); LLA (liver-lactose agar of Lapiere); BHI (brain heart infusion agar); LBS (Lactobacillus selective agar) and TSAYE (tryptone soya agar supplemented with 0.6% yeast extract). All agar media was supplied by Oxoid Chemicals with the exception of TPY agar. Plates were incubated in anaerobic jars (BBL, Oxoid) using CO_2 generating kits (Anaerocult A, Merck) for 2-5 days at 37°C.

Gram positive, catalase negative rod-shaped or bifurcated/ pleomorphic bacteria isolates were streaked for purity on to complex non-selective media (TPY). Isolates were routinely cultivated in TPY medium unless otherwise stated at 37°C under anaerobic conditions. Presumptive Bifidobacteria species were stocked in 40% glycerol and stored at -20° and -80°C.

Fermentation end-product analysis

Metabolism of the carbohydrate glucose and the subsequent organic acid end-products were examined using an LKB Bromma, Aminex HPX-87H High Performance Liquid Chromatography (HPLC) column. The column was maintained at 60°C with a flow rate of 0.6 ml/min (constant pressure). The HPLC buffer used was 0.01 N H_2SO_4. Prior to analysis, the column was calibrated using 10 mM citrate, 10 mM glucose, 20 mM lactate and 10 mM acetate as standards. Cultures were propagated in modified MRS broth for 1-2 days at 37°C anaerobically. Following centrifugation for 10 min at 14,000 g, the supernatant was diluted 1:5 with HPLC buffer and 200 µl was analysed in the HPLC. All supernatants were analysed in duplicate..

Biochemical and physiological characterisation

Biochemical and physiological traits of the bacterial isolates were determined to aid identification. Nitrate reduction, indole formation and expression of ß-galactosidase activity were assayed. Growth at both 15°C and 45°C and protease activity on gelatin were determined. Growth characteristics of the strains in litmus milk were also assessed.

Antibiotic sensitivity profiles

Antibiotic sensitivity profiles of the isolates were determined using the 'disc susceptibility' assay. Cultures were grown up in the appropriate broth medium for 24-48 h, spread-plated (100µl) onto agar media and discs containing known concentrations of the antibiotics were placed onto the agar. Strains were examined for antibiotic sensitivity after 1-2 days incubation at 37°C under anaerobic conditions.

Strains were considered sensitive if zones of inhibition of 1mm or greater were seen.

Isolation of Bifidobacteria sp.

Seven tissue sections taken from the human G.I.T. were screened for the presence of strains belonging to the *Bifidobacterium* genus. There was some variation between tissue samples as follows. Samples A (ileum) and E (appendix) had the lowest counts with approximately 10^2 cells isolated *per* gram of tissue. In comparison, greater than 10^3 cfu/g tissue were recovered from the other samples. Similar numbers of bacteria were isolated during the 'wash' and 'sample' steps with slightly higher counts in the 'sample' solutions of F (ileum) and G (ileal-caecal). Of those screened for tightly-adhering bacteria (homogenised), C (ileal-caecal) was the only tissue section that gave significant counts.

During the screening of some tissue sections, for example C and B, there was not a direct correlation between counts obtained during a dilution series. This would indicate that some growth factors, either blood or tissue derived, were being provided for the growth of the fastidious bacteria in the initial suspension which was subsequently diluted out.

Strain selection and characterisation

Approximately fifteen hundred catalase negative bacterial isolates from different samples were chosen and characterised in terms of their Gram reaction, cell size and morphology, growth at 15°C and

45°C and fermentation end-products from glucose. Greater than sixty percent of the isolates tested were Gram positive, homofermentative cocci arranged either in tetrads, chains or bunches. Eighteen percent of the isolates were Gram negative rods and heterofermentative coccobacilli.

The remaining isolates (twenty-two percent) were predominantly homofermentative coccobacilli. Thirty eight strains were characterised in more detail- 13 isolates from G; 4 from F; 8 from D; 9 from C; 3 from B and 1 from E. All thirty eight isolates tested negative both for nitrate reduction and production of indole from tryptophan.

Antibiotic sensitivity profiles

Antibiotics of human clinical importance were used to ascertain the sensitivity profiles of selected bifidobacteria. The bifidobacteria tested were sensitive to ampicillin, amoxycillin, ceftaxime, ceftriaxone, ciprofloxacin, cephradine, rifampicin, amikacin, gentamicin and chloramphenicol. They were also resistant to netilmicin, trimethoprim, nalidixic acid, cefuroxime, vancomycin and tetracycline.

Example 2

Bifidobacterium longum infantis in the treatment of ulcerative colitis

Acid Resistance

The first inhospitable site that a micro-organism reaches following human consumption is gastric acid. A key factor influencing bacteria is survival in gastric juice. The survival and growth of *Bifidobacterium longum infantis* strains 35612 and 35624 in a low pH environment were examined. The strains were routinely cultured in trypticase-peptone-yeast extract (TPY) medium at 37°C under strict anaerobic conditions (BBL Gas jars using the Merck Anaerocult A gas pak system) for 12-24h. Human gastric juice was obtained from healthy subjects by aspiration through a nasogastric tube (Mercy Hospital, Cork, Ireland). It was immediately centrigued at 13,000 g for 30 min. to remove all solid particles, sterilised through 0.45µm filters and 0.2µm filters and stored at 4°C. The pH and pepsin activity were measured prior to experimental use. Pepsin activity was measured using the quantitative haemoglobin assay (Guantam, S. and R.S. de la Motte. 1989. Proteolytic enzymes, a practical approach. Chapter 3. R.J. Beynon and J.S. Bond(eds.), IRL Press, Oxford University Press; Dawson, R.M.1969. pH and buffers. In Data for Biochemical Research p 138. R.M.Dawson, D.C. Elliot and K.M. Jones(eds), Clarendon Press, Oxford). Survival of the strains at low pH *in vitro* was investigated using the following assays:

(a) Cells were harvested from fresh overnight cultures, washed twice in phosphate buffer (pH 6.5) and resuspended in MRS broth adjusted to pH 3.5, 3.0, 2.5 and 2.0 (with 1N HCl) to a final concentration of approximately 10^6 cfu/ml. Cells were incubated at 37°C and survival measured at intervals of 5, 30, 60 and 120 min. using the plate count method.

The strains survived with no loss of viability at pH 3.5. At pH 2.5 there was a 3 log reduction over the 60 min. incubation period as depicted in Figs. 1 and 2.

Survival of strains of *Bifidobacterium* in human gastric juice.

Fresh overnight cultures were harvested, washed twice in buffer (pH 6.5) and resuspended in human gastric juice to a final concentration of 10^6 cfu/ml. Survival was monitored over a 30-60 min incubation period at 37°C. The experiment was performed using gastric juice at pH 1.2 (unadjusted) and pH 2.0 and 2.5 (adjusted using 1N NaOH).

Survival of the strains was increased in gastric juice at pH 2.0, when compared with gastric juice at pH 1.2. After 30 min. incubation no viable cells were recovered at either pH as shown in Table 2.

Table 2
Survival of *Bifidobacterium* sp. in human gastric juice *

STRAIN		pH	TIME (min) 0	5	30	60
	35612	1.2	7.56	0.00	0.00	0.00
		2.0	6.27	6.31	2.88	0.00
	35624	1.2	5.96	4.18	0.00	0.00
		2.0	6.33	6.32	0.00	0.00
	35652	1.2	6.16	3.78	0.00	0.00
		2.0	8.45	8.40	3.45	0.00
	35658	1.2	6.00	0.00	0.00	0.00
		2.0	7.89	6.45	0.00	0.00
	35687	1.2	6.68	0.00	0.00	0.00
		2.0	8.75	8.77	3.34	0.00
	BO	2.0	8.41	8.56	8.42	8.43
	10	2.0	8.39	8.56	4.64	0.00
	6.3	2.0	8.75	8.75	8.29	8.42
B. longum	6	2.0	8.15	8.02	0.00	0.00

* survival expressed as $\log_{10}$ cfu/ml

Example 3

Bile resistance

In the evaluation of the effectiveness of using lactic acid bacteria as beneficial members of the gastrointestinal tract, it is considered that resistance to bile acids is an important biological strain characteristic required for survival in this hostile environment and in addition they must not impinge on the health of the host by producing toxic compounds such as deoxycholic (DCA) and lithocholic acid (LCA) which have been implicated in a number of cytotoxic phenomena.

A number of *Bifidobacterium longum infantis* strains were streaked onto TPY agar plates supplemented with porcine bile (B-8631, Sigma Chemical Co. ltd., Poole) at concentrations of 0.3, 0.5, 1.0, 1.5, 5.0 and 7.5% (w/v) (Legrand-Defretin, R. *et al.*, Lipids 1991; 26 (8), 578-583). Porcine bile is the closest in composition to human bile with respect to bile salt/cholesterol and phospholipid/cholesterol ratios. Plates were incubated at 37°C under anaerobic conditions and growth was recorded after 24-48 h. Strain 35624 was found to be strongly bile resistant and grew to confluence at up to 5% porcine bile as shown in Table 3.

Table 3

Growth of *Bifidobacterium* sp. isolates in the presence of porcine bile

STRAIN	% (w/v) PORCINE BILE						
	0.0	0.3	0.5	1.0	1.5	5.0	7.5
35612	+	-	-	-	-	-	-
35624	+	+	+	+	+	+	-
35652	+	-	-	-	-	-	-
35658	+	+	+	+	-	-	-
35687	+	-	-	-	-	-	-

-, no growth; +, confluent growth

Human bile was obtained from several human gall bladders and sterilised at 80°C for 10 min. The bile acid composition of human bile was determined using reverse phase High Performance Liquid Chromatography (HPLC) in combination with a pulsed amperometric detector according to the method of Dekker, R.R. *et al.*, *Chromatographia*, 1991, 31 (11/12), 255-256. Human bile was added at a concentration of 0.3% (v/v). Freshly streaked cultures were examined for growth after 24 and 48h.

Strain 35624 was capable of growth in the presence of physiologically relevant human bile (0.3% (v/v)).

Growth of the strains was examined in the presence of individual conjugated and deconjugated bile acids. Under physiological conditions bile acids are often found as sodium salts. The strains were screened for growth on TPY agar containing the conjugated and deconjugated sodium salts of each of the following bile acids.

(a) *conjugated form*: glycocholic acid (GCA); glycodeoxycholic acid (GDCA); and glycochenodeoxycholic acid (GCDCA);
(b) *deconjugated form*: lithocholic acid (LCA); chenodeoxycholic acid (CDCA); deoxycholic acid (DCA) and cholic acid (CA). For each bile acid concentrations of 1, 3 and 5 mM were used. Growth was recorded after 24 and 48 h anaerobic incubation.

The five strains studied grew on agar medium supplemented with 5 mM GCA and GCDCA and on agar medium supplemented with 1 mM GDCA as shown in Table 4. Strain 35624 was resistant to concentrations of 5 mM LCA (data not shown) and strains 35612 and 35624 were capable of growth at concentrations of 5 mM CA as shown in Table 5. No growth was observed in the presence of 1 mM CDCA (data not shown).

Table 4

Growth of *Bifidobacterium* sp. isolates in the presence of glycine-conjugated bile acids

STRAIN	BILE ACIDS (mM)											
	GCDCA				GDCA				GCA			
	0	1	3	5	0	1	3	5	0	1	3	5
35612	+	+	+	+	+	+	+	+	+	+	+	+
35624	+	+	+	+	+	+	+	+	+	+	+	+
35652	+	+	+	+	+	+	+	+	+	+	+	+
35658	+	+	+	+	+	+	+	+	+	+	+	+
35687	+	+	+	+	+	+	+	+	+	+	+	+

-, no growth; +, confluent growth

GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; CGA, glycocholic acid.

Table 5

Growth of *Bifidobacterium* sp. isolates in the presence of unconjugated cholic acid (CA)

STRAIN	CHOLIC ACID (mM)			
	0	1	3	5
35612	+	+	+	+
35624	+	+	+	+
35652	+	+	-	-
35658	+	+	-	-
35687	+	+	-	-

-, no growth; +, confluent growth

Example 4

Antimicrobial Activity

Bifidobacterium species exert inhibitory effects on other bacteria by excluding long term colonisation by invasive pathogens. Their antagonistic activity is due to the production of acetic and lactic acid though fermentation (Scardovi, V. (1986) *Bifidobacterium* in Bergey's Manual of systemic bacteriology, Vol. 2, eds. Sheath, P.H., Main, N.S., Sharpe, M. and Holdt, J.G., Williams and Wilkins Publishers, Baltimore M.D.., p1418). Very few reports exist on

the production of antimicrobial compounds other than acids (Anand, S.K *et al.* Cult.Dairy Prods. 1985; J. 2, 21-23). Bacteriocins and other compounds may influence the survival of a bacterium in an ecological niche and allow them to effectively dominate fermenting ecosystems. Such a feature is a good trait for a probiotic strain.

The inhibitory spectra of various bifidobacterial strains was determined by the method of Tagg *et al.* (Tagg. J.R. *et al.* Bacteriol. Rev. 1976; 40, 722-756). Cell free supernatant was assayed for inhibitory activity against a wide range of Gram positive and Gram negative micro-organisms. Overlays of each indicator were prepared on agar plates and allowed to dry. Spots (5ml) of cell free supernatant were placed on the seeded plates, allowed to dry and the plates were incubated overnight.

It was observed that the strains were inhibitory to a wide range of *Staphylococcus*, *Pseudomonas*, *Coliform* and *Bacillus* sp. when testes on TPY medium. Zones of inhibition of up to 5.5mm were recorded against *Pseudomonas* and *Staphylococcus* and up to 7.0 mm surrounding *Bacillus* sp. as shown in Tables 6 and 7. However, when the deferred assays were performed on buffered TPY medium, zones of inhibition were not observed against any indicator strain. Therefore, inhibition appeared to be solely due to the presence of acid produced by the bifidobacteria.

Table 6

Inhibition of *Staphylococcus* strains by *Bifidobacterium* sp. on unbuffered medium*

	B. *longum* 1	B. *longum* 9	B. *longum* 10	63	35612	35624	35652	35658	35675	35678	35687
S. *aureus* MHS	1.5	2	1.5	3.5	1.5	1	2	2	1	2.5	1.5
S. *aureus* HC	1.5	1.5	2	2.5	2	1.5	2.5	2.	1.5	1.5	2
S. *aureus* 771	1.5	3	1.5	3	2	2	2.5	2	3	2	3.5
S. *aureus* 949	2	3.5	2.5	2	3	3.5	3	2.5	3.5	3.5	2.5
S. *aureus* 1018	1	3.5	1.5	1.5	2	3.5	1	3	3.5	2.5	2
S. *aureus* 1502	1.5	3.5	1	2	2.5	2.5	1.5	3	4	2.5	1.5
S. *aureus* 1505	3	4	3	2.5	2.5	3	2.5	4.5	5.5	5	5.5
S. *aureus* 1511	1	3.5	2	1.5	2	2.5	3	3.5	4	2.5	3
S. *aureus* 1522	1.5	3	2.5	1	2.5	1.5	2.5	2.5	3.5	3.5	3
S. *aureus* 1499	1.5	3.5	1.5	1.5	2	2	3	2	3.5	3.5	1.5
S. *aureus* 1963	2	3	2	2.5	3.5	3.5	3.5	3.5	2.5	3	2.5
S. *aureus* PRMM	1	3.5	1	1.5	1	3.5	2	2	3	2	2.5
S. *albus*	1	2	1.5	1	2	2.5	2	1.5	2	1.5	1
S. *carnosus*	1	1.5	2	2.5	2.5	2.5	2	2.5	2	1.5	1

* , values given are radii of inhibition zones in mm (distance from edge of producer colony to the edge of zone of inhibition)

Table 7

Inhibition of *Pseudomonas* and *Bacillus* strains by *Bifidobacterium* sp. on unbuffered medium*

	B. longum 1	*B. longum 9*	*B. longum 10*	63	35612	35624	35652	35658	35675	35678	35687
P. fluorescens HC.	1	2.5	1.5	1	1.5	2	3	2	1.5	2	2.5
P. fluorescens MHP	1.5	4.5	3.5	2	2.5	3.5	2.5	2.5	3.5	2	4
P. fluorescens DW	1.5	4	4	3.5	2.5	3.5	2.5	4.5	5.5	3.5	5
B. cereus	3	3	5	3	4	4	3.5	5	6	4.5	5.5
B. subtilis	2	2.5	5	2	3	6	3	6	7	3	6
B. circulans	1	2	4	1.5	2.5	1.5	2	3.5	4.5	2	4.5
B. thuringensis	2.5	3.5	5	3	3.5	4.5	4	5.5	6.5	4.5	5.5

*, values given are radii of inhibition zones in millimetres (distance from edge of producer colony to the edge of the zone of inhibition)

Example 5

Murine Feeding trial to investigate the ability of *Lactobacillus salivarius* subsp. *Salivarius* UCC 118 and *Bifidobacteria longum infantis* 35624 to alleviate the symptoms of Inflammatory Bowel Disease (IBD)

Background

A number of mouse models have recently been generated by either genetic or immunological means to study the mechanisms of IBD. One of these models involves the transfer of spleen or lymph node-derived $CD4^+$T lymphocytes from normal mice into severe combined immunodeficient recipient mice (SCID). It has been demonstrated that mice who receive purified $CD4^+$, $CD45RB^{high}$ T cells develop a wasting disease characterised by chronic intestinal inflammation which is more severe in the colon. In this study a control group of SCID mice was injected with $CD4^+$ $CD45RB^{high}$ and the mice developed a progressive wasting disease including hunched over appearance, piloerection of the coat, diarrhoea, weight loss and macro- and microscopic colon damage. A feeding trail was set up administering UCC 118 and strain 35624 (also referred to herein as UCC 35624) to determine if the symptoms of IBD could be modified in this model.

Bacterial strains

Lactobacillus salivarius subsp. *salivarius* UCC 118 and *Bifidobacterium longum infantis* UCC 35624 were isolated from the ileal-caecal region of an adult human as described in Example 1. In this example, spontaneous rifampicin and streptomycin resistant derivatives

of the strains were generated by plating cells, previously grown overnight and subsequently washed in quarter strength Ringer's solution, on MRS and TPY agar containing 50μg/ml rifampicin (Sigma) respectively and MRS containing 400μg/ml streptomycin (Sigma). Plates were incubated for 2 days at 37°C anaerobically. The resulting antibiotic resistant derivatives were determined to be otherwise phenotypically similar to the parent strain. This selectable trait enabled the strains to be readily enumerated following gut transit.

Animals and maintenance

Donor mice (C57BL/6 x BALB/c)F1 were purchased from Simosen Laboratories (Gilroy, CA) and maintained at the University of California-Los Angeles vivarium in ventilated cage racks (Thoren caging systems, Hazelton, PA) under specific pathogen free (SPF) conditions. CB-17 SCID mice were bred in ventilated cage racks originally obtained from the University of California-Los Angeles SCID core facility. The mice were reduced flora(RF) mice rather than germ free and acting as the recipient mice (Aranda R. *et al.* J. *of Immunol.* 1997; 158(7), 3464-3473)

Eight week old, female CB-17 (SCID) mice were housed in pairs in filter top cages in ventilated racks. The mice were divided into four groups Group A: consumed 10% skim milk, Control; Group B: consumed *Lactobacillus salivarius* UCC 118, Group C: consumed *Lactobacillus salivarius* UCC 118 and *Bifidobacterium longum* UCC 35624 9 (1:1 ratio); Group D: consumed *Bifidobacterium longum* UCC 35624. UCC 118 and UCC 35624, which were grown overnight in MRS broth and MRS broth supplemented with 0.05% cysteine (Sigma) respectively, were washed in PBS, resuspended in skim milk (10% (v/v))

and administered in the otherwise sterile drinking water (PBS). The mice in each respective group received $2.55x10^8$ cfu/ml of UCC 118 and $2.35x10^8$ cfu/ml of UCC 35624 daily for the duration of the feeding period. Control mice received sterile milk diluted in sterile phosphate buffered saline (PBS) and were maintained under identical conditions as the test groups.

Experimental design

All CB-17 mice were administered their respective feed according to their grouping for 2 days prior to injection with the $CD4^+$ CD45 RB^{high} cells. The sorted donor lymphocytes ($3-4 \times 10^5$) were represented in 200µl of sterile PBS and injected i.p. into the recipient CB-17 SCID mice. All mice were weighed initially, then twice weekly thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat and diarrhoea.

Evaluation of the effects of the administered probiotics on the numbers of indigenous bacteria culturable from mouse faeces.

The influence exerted by the administered UCC 118 and UCC 35624 when either administered alone or in combination with each other, on the microflora of the CB-17 SCID murine gut was investigated. Faecal samples were collected from each mouse weekly, weighed and resuspended in 10ml PBS. The samples were then serially diluted in PBS and either pour plated or spread plated in appropriate dilutions on appropriate media in duplicate. The following bacterial groups were enumerated: lactobacilli; bifidobacteria; enterococci; bacteroides and coliforms. The selective media used were; de Mann Rogosa &

Sharpe(MRS) agar; MRS agar supplemented with 0.2% lithium chloride(BDH), 0.3% sodium propionate (Fluke chemie), 0.5% cysteine hydrochloride (Sigma), and 5% sheep's blood; Slanetz and Bartley agar; Wilkins and Chalgren agar supplemented with anaerobic supplement SR108 and 5% horse blood; and Violet Red Bile Agar.(All Oxoid unless otherwise stated). VRBA and Slanetz and Bartley plates were incubated aerobically for 24 and 48 h respectively. All other plates were incubated anaerobically for 48 h at 37°C.

Enumeration of culturable indigenous flora from specific segments of the CB.17 SCID murine G.I.T.

After the feeding period all mice were sacrificed and dissected. Segments of the ileal-caecal region, small intestine, and the large intestine were removed. A peripheral lymph node (PLN), mesenteric lymph node (MLN) and a piece of the spleen were also taken. All tissues were weighed before being resuspended in 10 ml of PBS. Samples were then homogenised and serially diluted in PBS and either spread plated or pour plated in appropriate dilutions on appropriate media in duplicate. The bacterial groups were enumerated the same as those enumerated in the faecal analysis and samples were incubated as described previously.

Preparation of intraepithelial and lamiinapropria lymphocytes

The isolation of the mucosal lymphocytes was carried out according to the method of Aranda, R. *et al* ((1997) *supra*).

Flow cytometric analysis of lymphocyte populations.

The analysis was conducted as described by Aranda, R. *et al.* ((1997) *supra*)

Preparation of tissue for histopathological analysis.

Tissue samples were taken from the small intestine, large intestine, and ileal caecal region and fixed in 10% formalin. The procedure was as described in Aranda, R. *et al.* ((1997) *supra*)

It was observed from the experiment carried out that, consistent with previous results, the SCID mice reconstituted with $CD4^+$ $CD45RB^{high}$ T lymphocytes and consuming skim milk alone (control) developed a progressive wasting disease, identified by their significant weight loss. Disease became apparent at about 2 and a half to three weeks and the sick mice characteristically manifested a hunched over appearance, piloerection of their coat, and loose stool. One of the mice in the control group(mouse 4) died after 25 days and mice 1, 2, 3 and 5 showed a -20%, -25%, -21% and –35% percentage weight change respectively as depicted in Figs. 3 and 4.

CB-17 SCID mice consuming UCC 118 alone gave a similar result as the controls with the characteristic weight loss. Mouse 3 died after 14 days, and mice 4, 5 and 6 showed a –15%, -25% and –28% percentage weight change respectively (data not shown). The mice consuming a combination of UCC 118 and UCC 35624 were found to have a marked

improvement on the control mice. These mice did not lose as much weight as the control mice over the feeding period. Even after 35 days three of the mice in this group showed little percentage weight change. (Figs. 5 and 6). Two of the mice in this group showed a weight loss only after about 30 days whereas control mice showed weight loss at 14 days (Figs. 3 and 4).

Mice consuming UCC 35624 alone appeared in good health and again weight loss when compared to the controls was considerably less (Figs. 7 and 8). It can be concluded therefore that consumption of UCC 35624 either alone or in combination with UCC 118 alleviates the symptoms of inflammatory bowel disease.

Table 8 is a summary of experimental data for the study on the treatment of CD45RB colitis induced CB17 and SCID mice with a cocktail of UCC 118 and UCC 35624.

It was found in the studies that the mice were successfully reconstituted with lymphocytes, the lymphocytes having been derived from the donor model (data not shown).

Example 6

Lactobacillus salivarius subsp. *salivarius* (UCC 118) was used in a pilot trial in IL-10 knockout mice. For a period of 16 weeks, ten mice were fed UCC 118 (test group) while 10 mice were fed the control product (control group).

Microbial counts were performed weekly from stool samples. At 16 weeks, mice were sacrificed and microbial numbers were counted from within the colon, caecum and ileum. The most significant observations included decreased coliform and *Clostridium perfringens* numbers from mice fed UCC 118.

The mortality and incidence of cancer is:

	Control	Test
Mortality	20%	0%
Cancer	50%	10%

Murine gastrointestinal tissue was dissected and graded for inflammation. Inflammatory scores were assessed by two independent histologists. There were substantial reductions in these inflammatory scores at all sites examined (colon, caecum and ileum) in mice fed UCC 118 compared with mice fed the control product. This indicates a reduction in disease severity in mice consuming the probiotic product.

In general, the microbial counts were increased in mice with the higher inflammatory scores in both the caecum and colon.

Coliforms ($p=0.035$), enterococci ($p=0.0270$) and bacteroides ($p=0.068$) numbers were increased in mice who developed cancer ($n=6$) compared with those who were cancer free ($n=14$).

In conclusion, consumption of UCC 118 results in a significant modulation of the gut flora and an improvement in mortality rate, cancer incidence and disease score.

Table 8

Treatment of CD45RB colitis induced CB 17 SCID mice with a cocktail of *Lactobacillus salivarius* UCC 118 and Bifidobacteria.

Organ	Mouse 1 Untreated (RB hi cells + skimmed milk)	Mouse 2 Untreated (RB hi cells + skimmed milk)	Mouse 3 Cocktail Treated	Mouse 4 Cocktail Treated	Mouse 5 Cocktail Treated	Mouse 6 Cocktail Treated
% weight loss	31.25	21.74	14.50	14.05	21.88	11.18
Final Appearance	looks ill	very ill	very healthy	slightly ill	healthy	healthy
Stool Appearance	very mushy	very mushy	mushy	solid	semi solid	semi solid
Colon Appearance	thickened	very thickened	slightly thickened	slight proximal thickening	slightly thickened	slight proximal thickening
No. SIEL	100,000	200,000	0	0	512,000	28.000
No. LIEL	25,000	72,000	100,000	50,000	384,000	96.000
No. SLPL	200,000	100,000	264,000	200,000	640,000	104.000
No. LLPL	96,000	256,000	160,000	160,000	256,000	160.000
No. MLN	0	N/A	81,900	N/A	28,800	N/A
No. PLN	0	192,000	0	120,000	64,000	0
Spleen # Lymphos.	960,000	512,000	640,000	640,000	512,000	6.400.000
CD3+/H-2Kb+ Flow Correction %						
No. SIEL	62,000	114,000	0	0	450,560	17.920
No. LIEL	21,250	48,960	74,800	38,000	345,600	65.280
No. SLPL	74,000	42,000	158,400	136,000	384,000	66.560
No. LLPL	67,200	161,280	115,200	108,000	184,320	108.800
No. MLN	0	N/A	130,000	N/A	64,000	N/A
No. PLN	0	126,720	0	87,600	54,400	0
Spleen	518,400	102,400	211,200	307,200	230,400	4.480.000
UCC 118 bacterial counts (*per* biopsy) *post mortem*						
SI	0	0	1,200	0	0	0
LI	0	0	>30,000	>30,000	100	11.600
Caecum	0	0	>30,000	>30,000	>30,000	>30.000
Spleen	0	0	0	1,350	0	0
Colon Pathological Scoring						
A (0-3)	-	1.0	1.0	2.0	-	-
B (0-2)	-	1.5	1.0	1.0	-	-
C (0-3)	-	2.5	1.0	2.0	-	-
D (0-3)	-	2.0	3.0	3.0	-	-
E (1-3)	-	1.0	1.0	2.0	-	-
Remarks						
Total Score	-	8.0	7.0	10.0	-	-

A: Degree of inflammatory infiltrate; B: Mucin depletion; C: Epithelial hyperplasia; D: No. of TEL in the crypts; E: No. of inflammatory foci *per* high power fields.

CLAIMS: -

1. A strain of *Bifidobacterium longum infantis* isolated from resected and washed human gastrointestinal tract which is capable of combating the effects of inflammatory bowel disease, said capability being maintained in the presence of physiological concentrations of human bile and human gastric juice.

2. A strain of *Bifidobacterium longum infantis* according to Claim 1, wherein the capability of combating the effects of inflammatory bowel disease is measured by measuring a reversal of a wasting disease induced in severe combined immunodeficient recipient mice (SCID) which have been administered purified $CD4^+$, $CD45RB^{high}$ T cells.

3. A strain of *Bifidobacterium longum infantis* according to Claim 1 or 2, which has inhibitory activity against a broad range of Gram positive and Gram negative bacteria.

4. A strain of *Bifidobacterium longum infantis* according to Claim 3, wherein the strain exhibits a broad-spectrum of activity against bacteria including *Staphylococcus*, *Pseudomonas*, *Coliform* and *Bacillus* species.

5. A strain of *Bifidobacterium longum infantis* according to any preceding claim, which is *Bifidobacterium longum infantis* strain UCC 35624 or a mutant or variant thereof.

6. A formulation which comprises a strain of *Bifidobacterium longum infantis* according to any one of Claims 1-5.

7. A formulation according to Claim 6, which comprises two or more of said strains of *Bifidobacterium longum infantis*.

8. A formulation according to Claim 6 or 7, which comprises a strain of *Lactobacillus salivarius* isolated from resected and washed human gastrointestinal tract which inhibits a broad range of Gram positive and Gram negative micro-organisms and which secretes a product having antimicrobial activity into a cell – free supernatant, said activity being produced only by growing cells and being destroyed by proteinase K and pronase E, the inhibitory properties of said strain and its secretory products being maintained in the presence of physiological concentration of human bile and human gastric juice.

9 A formulation according to Claim 8, wherein the strain of *Lactobacillus salivarius* is *Lactobacillus salivarius* strain UCC 118 or a mutant or variant thereof.

10. A strain of *Bifidobacterium longum infantis* according to any one of Claims 1-5 or a formulation according to any one of Claims 6-9 for use in foodstuffs.

11. A strain of *Bifidobacterium longum infantis* according to any one of Claims 1-5 or a formulation according to any one of Claims 6-9 for use as a medicament.

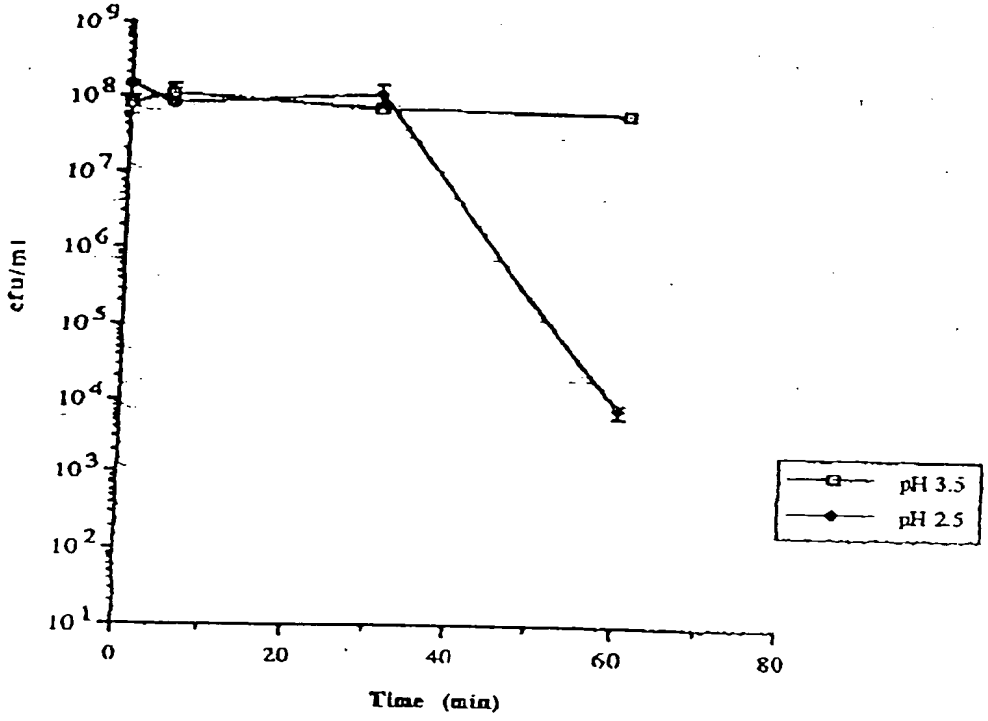
12. A strain of *Bifidobacterium longum infantis* according to any one of Claims 1-5 or a formulation according to any one of Claims 6-9 for use in combating the effects of inflammatory bowel disease.

ANNE RYAN & CO.
AGENTS FOR THE APPLICANTS

Abstract

Bifidobacterium longum infantis in the treatment of inflammatory bowel disease

A strain of *Bifidobacterium longum infantis* was isolated from resected and washed human gastrointestinal tract which is capable of combating the effects of inflammatory bowel disease. This capability is maintained in the presence of physiological concentrations of human bile and human gastric juice. The capability of combating the effects of inflammatory bowel disease can be measured by measuring a reversal of a wasting disease induced in severe combined immunodeficient recipient mice (SCID) which have been administered purified $CD4^+$, $CD45RB^{high}$ T cells. Two or more strains of *Bifidobacterium longum infantis* can be used in foodstuffs and medicaments optionally in the presence of a strain of *Lactobacillus salivarius*, such as *Lactobacillus salivarius* strain UCC 118.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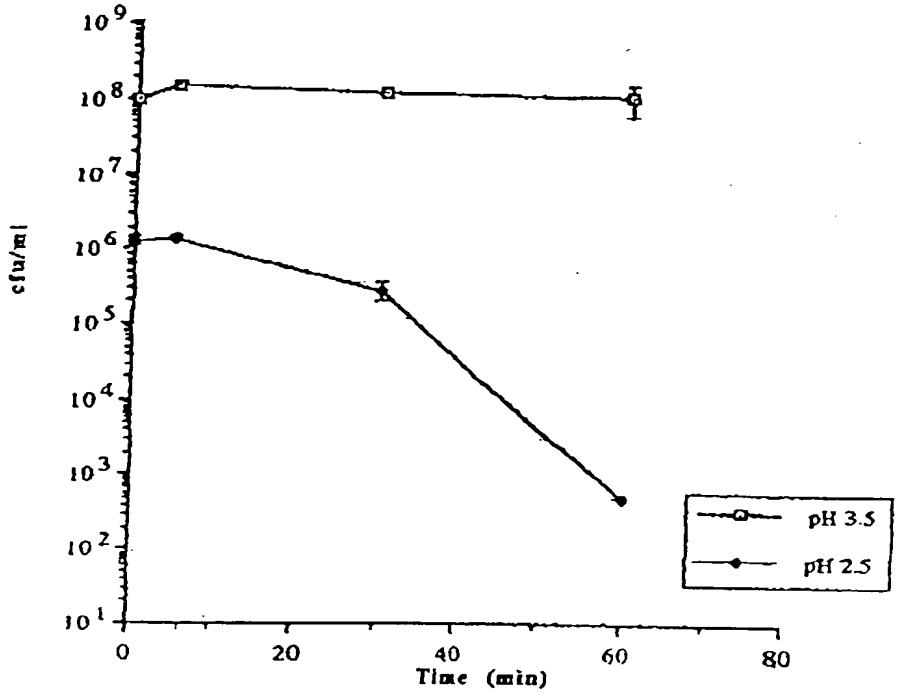
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
cfu/ml
0
20
40
60
80
Time (min)
pH 3.5
pH 2.5

FIG. 1


10^9
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10^4
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cfu/ml
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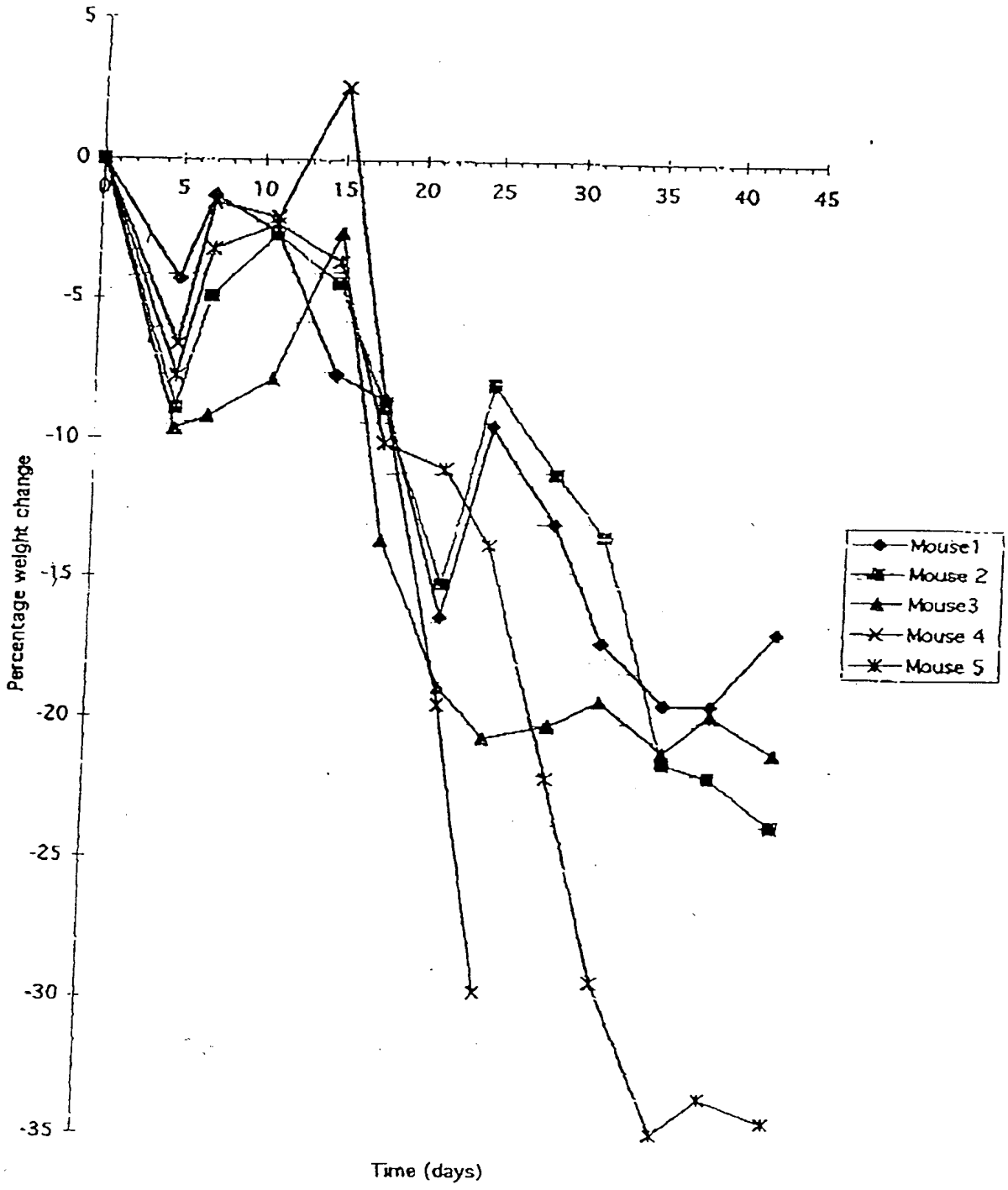
20
40
60
80
Time (min)
pH 3.5
pH 2.5

FIG. 2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0
-5
-10
-15
-20
-25
-30
-35
0
5
10
15
20
25
30
35
40
45
Percentage weight change
Time (days)
Mouse1
Mouse 2
Mouse3
Mouse 4
Mouse 5

FIG. 3

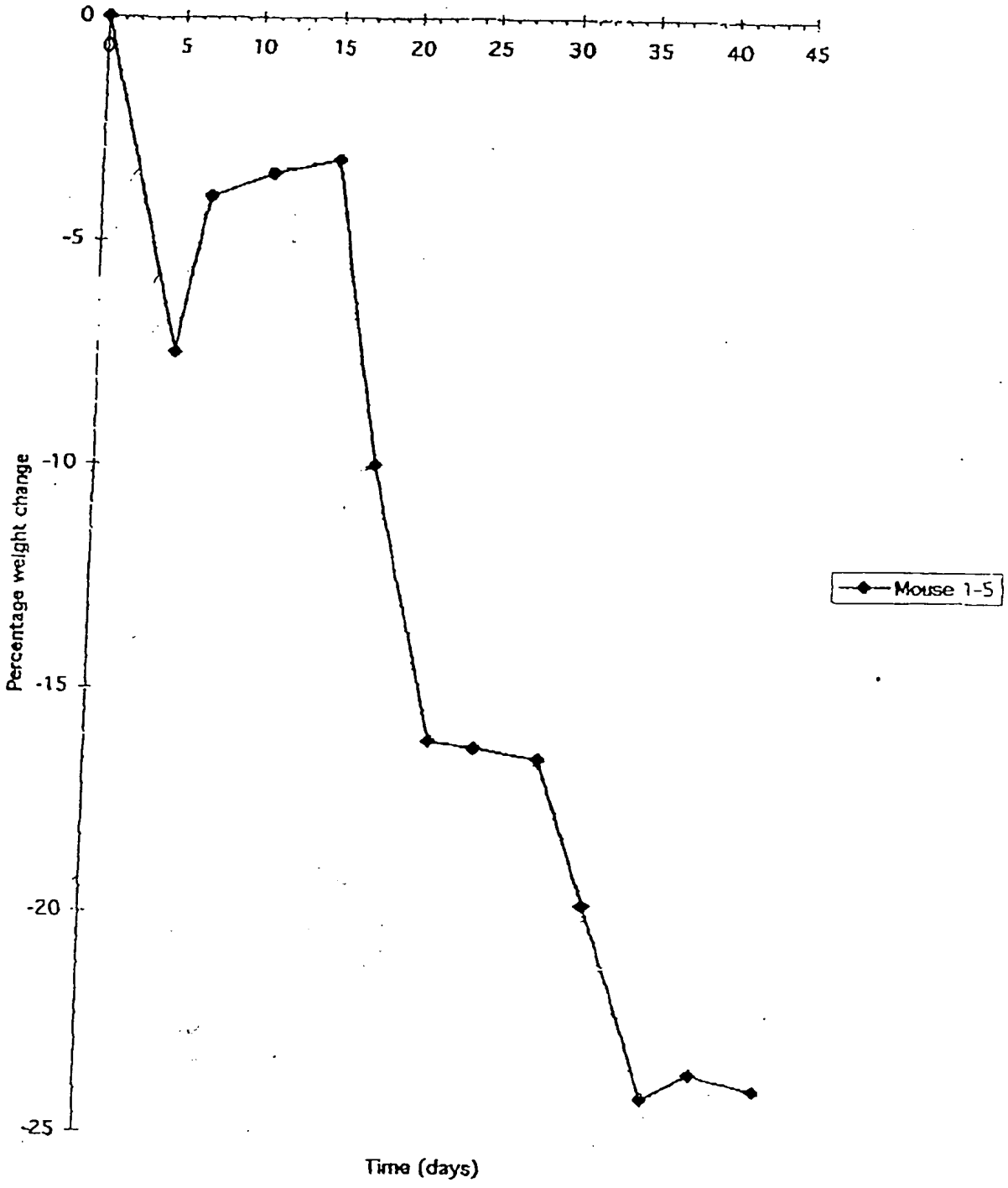
0
0
5
10
15
20
25
30
35
40
45
-5
-10
-15
-20
-25
Percentage weight change
Time (days)
Mouse 1-5

FIG. 4

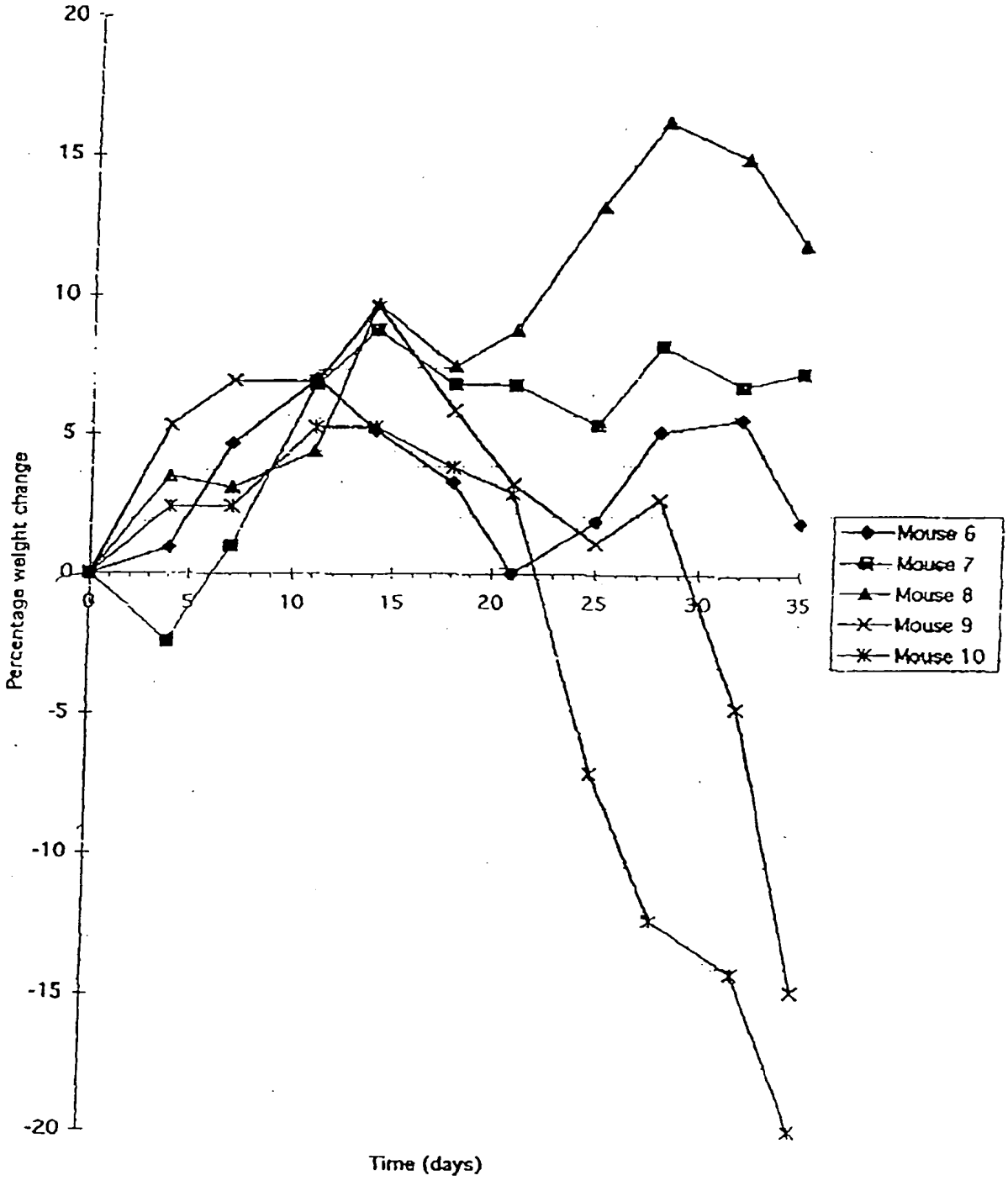
20
15
10
5
0
-5
-10
-15
-20
0
5
10
15
20
25
30
35
Percentage weight change
Time (days)
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10

FIG. 5

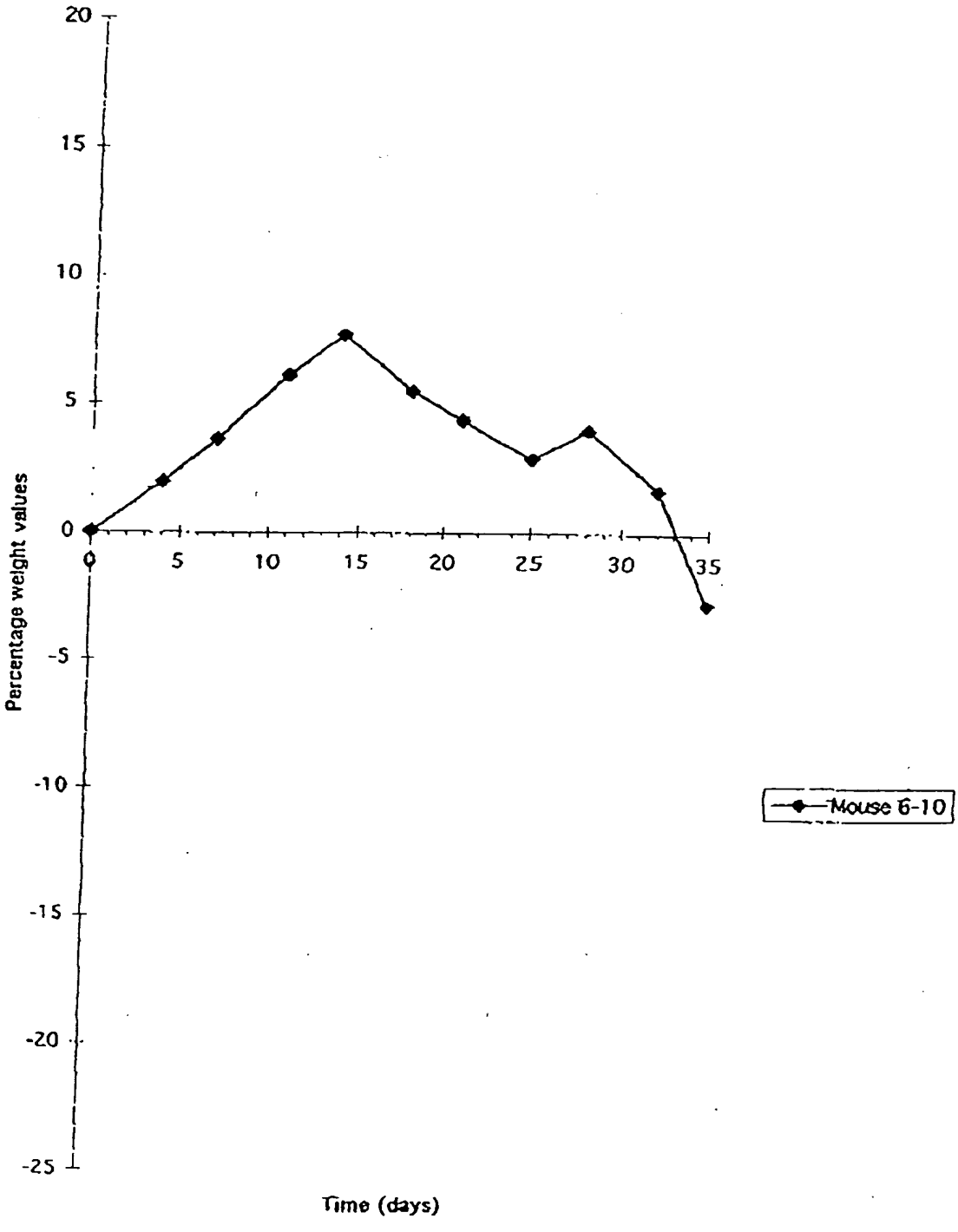
20
15
10
5
0
-5
-10
-15
-20
-25
0
5
10
15
20
25
30
35
Percentage weight values
Time (days)
Mouse 6-10

FIG. 6

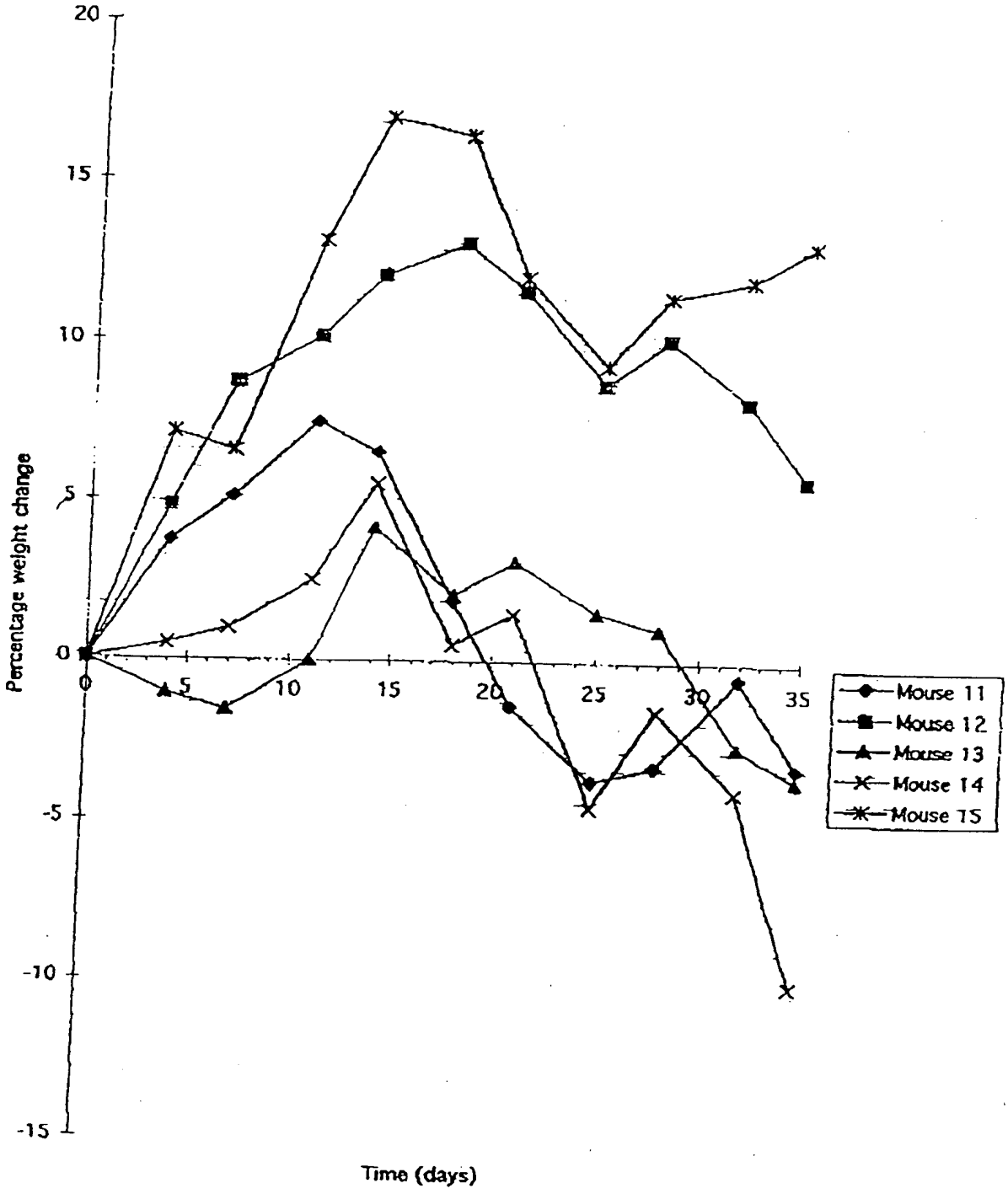
20
15
10
5
0
-5
-10
-15
Percentage weight change
0
5
10
15
20
25
30
35
Time (days)
Mouse 11
Mouse 12
Mouse 13
Mouse 14
Mouse 15

FIG. 7

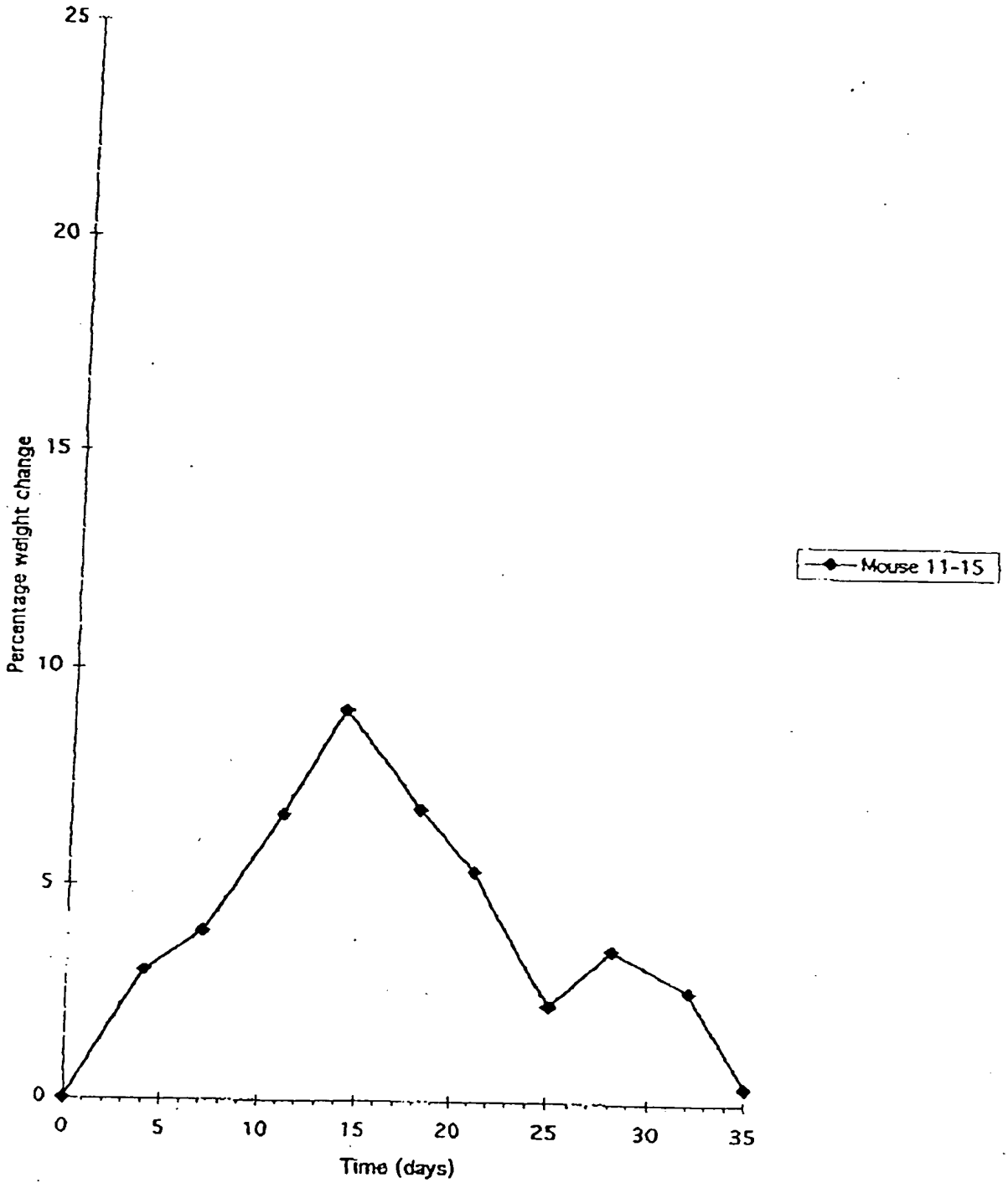
25
20
15
10
5
0
Percentage weight change
0
5
10
15
20
25
30
35
Time (days)
Mouse 11-15

FIG. 8